

EFFECTOR RESPONSE OF THE ASPARTATE TRANSCARBAMOYLASE
FROM WILD TYPE *Pseudomonas putida* AND A MUTANT
WITH 11 AMINO ACIDS DELETED
AT THE N-TERMINUS OF PYR B

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Like its enteric counterpart, aspartate transcarbamoylase (ATCase) from *Pseudomonas putida* is a dodecamer of two different polypeptides. Unlike the enterics, the *Pseudomonas* ATCase lacks regulatory polypeptides but employs instead inactive dihydroorotases for an active dodecamer. Previous work showed that PyrB contains not only the active site but also the effector binding sites for ATP, UTP and CTP at its N-terminus. In this work, 11 amino acids were deleted from the N-terminus of PyrB and the ATCase with the truncated protein was expressed in *E. coli pyrB⁻* and purified. The wild type enzyme was similarly treated. Velocity-substrate plots without effectors gave Michaelis-Menten kinetics in all cases. Deleting 11 amino acids did not affect dodecameric assembly but altered effector responses. When carbamoylphosphate was varied, the mutant enzyme was inhibited by UTP while the wild type enzyme was activated 2-fold. When the aspartate was varied, CTP had no effect on the mutant enzyme but strongly inhibited the wild type enzyme.

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INTRODUCTION

Pseudomonads

Pseudomonads are famous both for their omnivorous nutritional and metabolic versatility, and also for their wide environmental distribution. They play very important roles in chemical transformations of organic materials (10). Also, *Pseudomonas* species have achieved research prominence in microbiology in particular *P. aeruginosa* as it is associated with human infection, and because of its biochemical diversity. Pseudomonads also have strong relations with the environment because these bacteria interact with plants and play key roles in biodegradation of natural and man-made accumulation of toxic chemicals. Pseudomonads are a major group of chemoorganotrophic aerobic Gram-negative rods that lack a fermentative metabolism (7).

The genus *Pseudomonas* comprises a vast diverse group of bacteria. *Pseudomonas putida*, the subject of this thesis, is one of the fluorescent pseudomonads and can be found in a variety of natural environments. *P. putida* is a Gram-negative, aerobic, fluorescent pseudomonad, which grows optimally at around 30°C. Like other pseudomonads, *P. putida* thrives in moist environments. Its isolation from clinical specimens is an unusual event and is considered to have uncertain pathogenic significance. In immunocompromised patients, *P. putida* has been found to cause septicemia and septic arthritis (2,20).

Pyrimidine Pathway

The pyrimidine biosynthetic pathway is important for all organisms because it provides the building blocks namely UTP and CTP for ribonucleic acid (RNA), and dCTP and dTTP for deoxyribonucleic acid (DNA). There are two main pathways to

synthesize the RNA and DNA precursors. The first one is the pyrimidine biosynthetic pathway and the second is the pyrimidine salvage pathway. The former is a series of nine reactions, which lead to the formation of uridine-5-triphosphate (UTP) and cytidine-5-triphosphate (CTP). The salvage pathway is required for the recycling of UMP and CMP, the degradation products of mRNA turnover. The salvage pathway is also required for the feeding of exogenous nucleosides and bases to mutants of the biosynthetic pathway. At any time during the growth phase, the biosynthetic and salvage pathways each contribute 50% of the RNA synthesized. Thus, the salvage pathway maintains a metabolic balance in the cell.

Almost all organisms can make the purine and pyrimidine nucleotides by the *de novo* biosynthetic pathways. Pyrimidines are not synthesized as nucleotide derivatives. Instead, the pyrimidine ring system is completed before a ribose-5-P moiety is attached. Only two precursors, carbamoylphosphate and aspartate, contribute atoms to the six-membered pyrimidine ring.

The *de novo* pathway for pyrimidine biosynthesis is universal and virtually identical in all organisms (figure 1). Even though the sequence of events is similar in all organisms, the regulation of these events differs from one organism to another. The pathway, in general, is controlled at the level of enzyme activity by feedback inhibition and activation, and at the level of enzyme synthesis by repression and attenuation. In *E. coli* K-12, six consecutive, unlinked genes and small operons designated as *carAB*, *pyrBI*, *pyrC*, *pyrD*, *pyrE*, and *pyrF* encode the six enzymes of the pyrimidine pathway to UMP. This pathway is responsible for the synthesis of UMP, which is a precursor of all pyrimidine nucleotides. As mentioned above the pathway also provides the precursors for

deoxyribonucleotide biosynthesis, leading to the formation of dCTP and ultimately, dTTP (27).

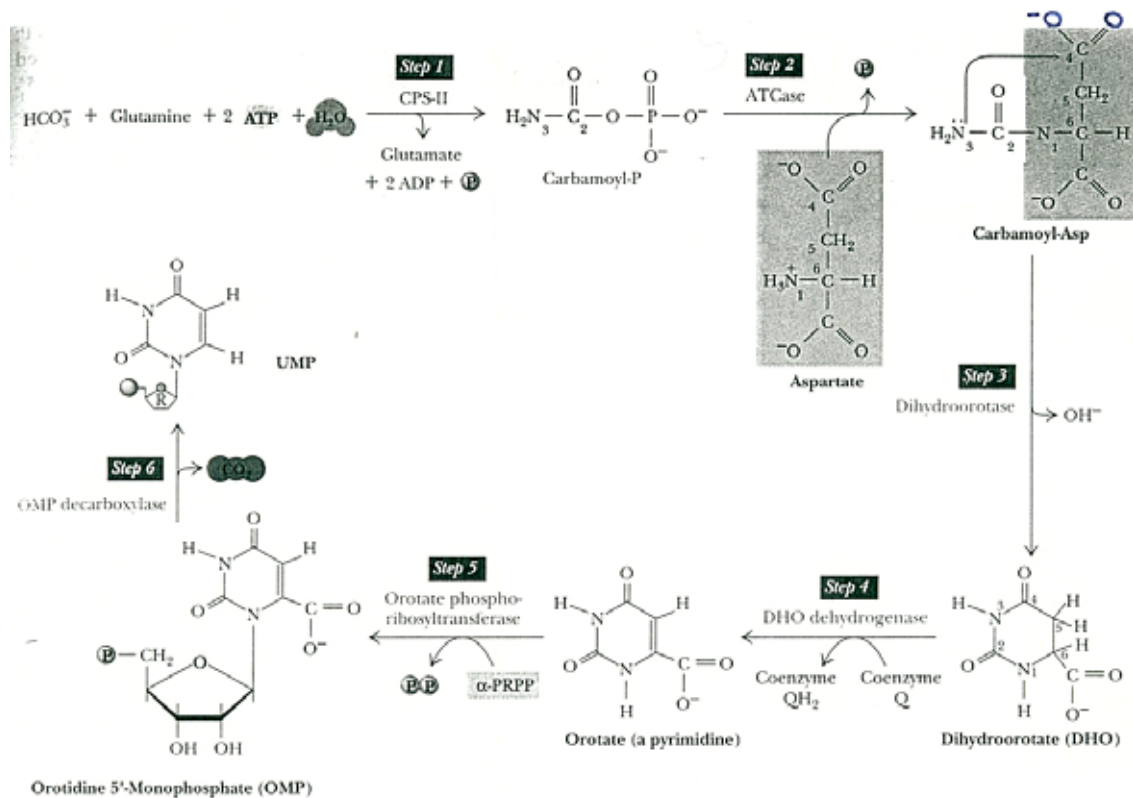


Figure 1. The *de novo* pyrimidine biosynthetic pathway to UMP.

The first enzyme in the *de novo* pathway is encoded by the operon *carAB*. This enzyme is called carbamoylphosphate synthetase (CPSase). In the enteric bacteria it functions in two biosynthetic pathways those of pyrimidines and arginine. Synthesis of this enzyme is controlled by cumulative repression by both arginine and a cytidine nucleotide, both of which are end products of their respective pathways (24, 28).

The enzyme that catalyzes the first reaction unique to pyrimidine biosynthesis is encoded by the second operon (*pyrBI*). This enzyme is designated aspartate transcarbamoylase, and it is involved in the carbamoylation of the amino group of carbamoylphosphate to produce carbamoylaspartate. This is the first committed step in

the pyrimidine pathway, which converts the precursors carbamoylphosphate and aspartate into carbamoylaspartate (CAA) and P_i .

ATCase is a highly conserved feature of the pathway; variations have been reported in both its regulation and size. In 1969, these variations led to a classification system proposed by Bethell & Jones (4).

The synthesis of ATCase is controlled by attenuation and its activity is controlled by allosteric inhibition by CTP and activation by ATP (12). CAA is converted next to dihydroorotate by the enzyme dihydroorotase (DHOase). This enzyme is encoded by *pyrC* gene. Next, the product of this reaction is oxidized to orotate. This step is catalyzed by the enzyme dihydroorotate dehydrogenase, which is encoded by the *pyrD* gene. Then, orotate combines with the phosphoribosyl group of 5-phosphoribosyl-1-pyrophosphate (PRPP) to form orotidine-5-monophosphate (OMP), and this is the first pyrimidine nucleotide produced (11). The enzyme that catalyzes this step is orotate phosphoribosyltransferase, which is encoded by *pyrE* gene. OMP is then decarboxylated to uridine-5-monophosphate (UMP). This step is catalyzed by OMP decarboxylase, which is encoded by the *pyrF* gene. UMP is phosphorylated to UDP using ATP as the source of phosphate. This enzyme, UMP kinase, is highly specific for UMP. Next, uridine-5- diphosphate is phosphorylated to UTP, again using ATP, by the enzyme nucleoside diphosphokinase. The last step in this pathway is the production of CTP from UTP. This step is catalyzed by the enzyme CTP synthetase, which is encoded by *pyrG* gene. Glutamine serves as the amino donor in this reaction(26).

Aspartate Transcarbamoylase (ATCase)

Bacterial ATCases have been divided into three classes based on molecular masses of the holoenzyme (4) (Fig.2). Class A ATCases are dodecameric (Fig 4) with a molecular mass of approximately 480 kDa. This class of ATCases is the largest and is sensitive to allosteric effectors. The genes for class A enzymes were originally cloned and sequenced from *Pseudomonas aeruginosa* (39) and *P. putida* (33).

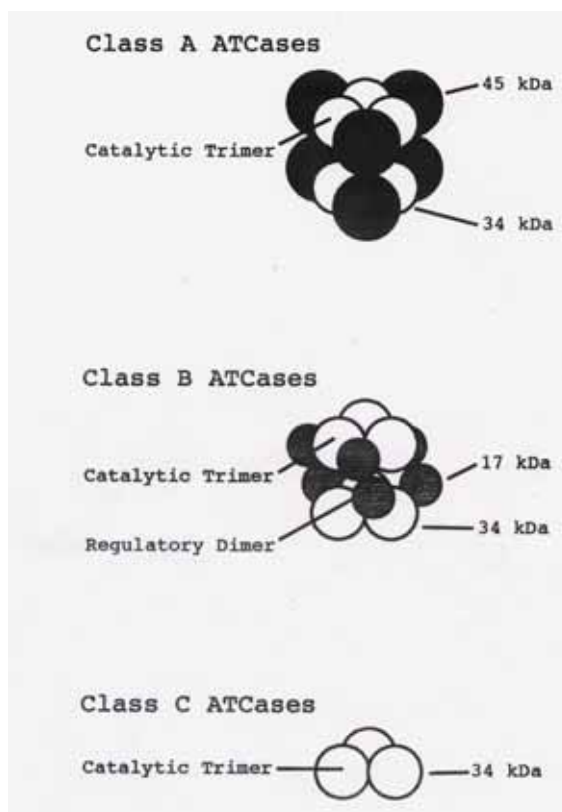


Figure 2. The three classes of bacterial ATCases.

The *Pseudomonas* ATCase belongs to class A ATCase (Fig 3). Members of this class have large ATCases with a molecular mass of 484 kDa. In *Pseudomonas* ATCase, unlike class B, both catalytic and regulatory sites reside in the 34 kDa polypeptides (9, 21, 22). The nucleotide sequence of the *P. putida* ATCase has been determined. The *P. putida* genes are arranged in an operon with three open reading frames (ORFs), a 5' ORF

corresponding to the *pyrR* gene (23), the middle ORF corresponding to the *pyrB* gene and a downstream ORF with significant sequence similarity to the *pyrC* gene encoding dihydroorotase (DHOase). In *P. putida*, the *pyrB* gene is 1005bp and is translated to 334 amino acids encoding polypeptide of 34.5 kDa (33). The *pyrC* gene with 30% sequence similarity to the *E. coli* DHOase, has 1275bp encoding 424 amino acids giving a polypeptide of 44.2 kDa. Because this polypeptide is catalytically inactive, it is designated PyrC' (DHOase-like) (33). The PyrC' polypeptides are required for the activity of the holoenzyme. It was expected that the PyrC' functioned as a source of nucleotide binding sites but Bergh & Evans, 1993 and Schurr *et al.*, 1995 showed that the nucleotide binding sites were located on the PyrB polypeptides.

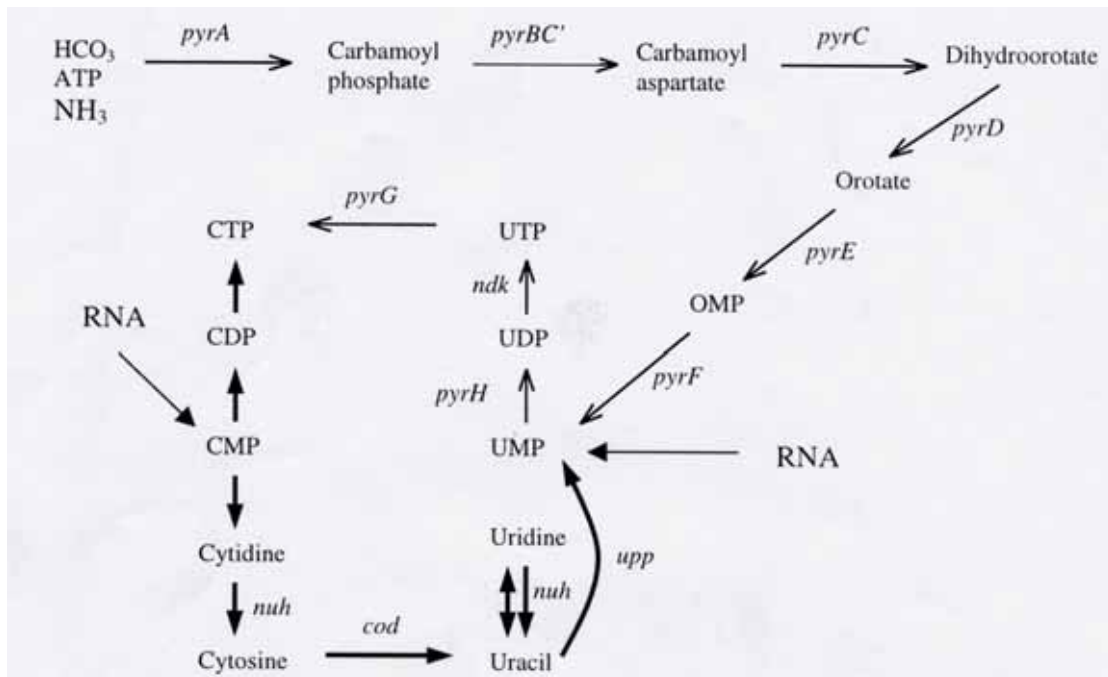


Figure 3. The pyrimidine pathway in *Pseudomonas*. Heavy lines denote salvage pathways.

The *Pseudomonas* holoenzyme is inhibited by all triphosphates, pyrophosphate, and orthophosphate at limiting concentration of carbamoylphosphate. The ATCase of

Pseudomonas was thought to be a dimer (1) until Schurr et al. 1995 provided the DNA sequence. Adair and Jones (1972) suggested that the ATCase from *P. fluorescens* was composed of two identical subunits, each with a molecular weight of 180 kDa. Recent studies on the ATCase of *P. putida* and *P. aeruginosa* show that there is no separate regulatory gene, but that the first gene in the sequence (*pyrB*) solely encodes ATCase, with shared catalytic and regulatory activities. The second gene (*pyrC'*) encodes an inactive DHOase (Schurr Ph.D. Dissertation, UNT, 1992). The gene for the active DHOase was cloned and sequenced by Dayna Brichta from *P. aeruginosa*. Class B enzymes are dodecamers like those of class A ATCases, but they have a smaller molecular mass. Class B ATCases have an average molecular mass of 310 kDa .

E. coli ATCase (Fig 4) is a typical class B enzyme. It consists of six copies of two polypeptides, catalytic chains of 34 kDa and regulatory chains of 17 kDa for an overall structure $2c_3:3r_2$ (16).

The *E. coli* ATCase has been the most studied (Fig 5). The catalytic activity of ATCase is dependent on the formation of an active site from half-sites and separate subunits (17, 31). The catalytic polypeptide is composed of two domains, the polar domain, at the N-terminus, binds carbamoyl phosphate and the equatorial domain at C-terminus, binds aspartate. Each active enzyme is a trimeric protein containing three active sites. *E. coli* and the enteric bacteria contain a second polypeptide, with regulatory properties only, which efficiently binds nucleotide effectors (ATP, and CTP) when regulating enzymatic activity. In these bacteria, the *pyrBI* genes lie beside each other and are expressed coordinately. The catalytic polypeptides are encoded by the *pyrB* gene and the regulatory polypeptides are encoded by the *pyrI* gene. These polypeptide chains

together form a dodecamer, containing two trimeric catalytic subunits and three dimeric regulatory subunits ($2c_3 : 3r_2$).

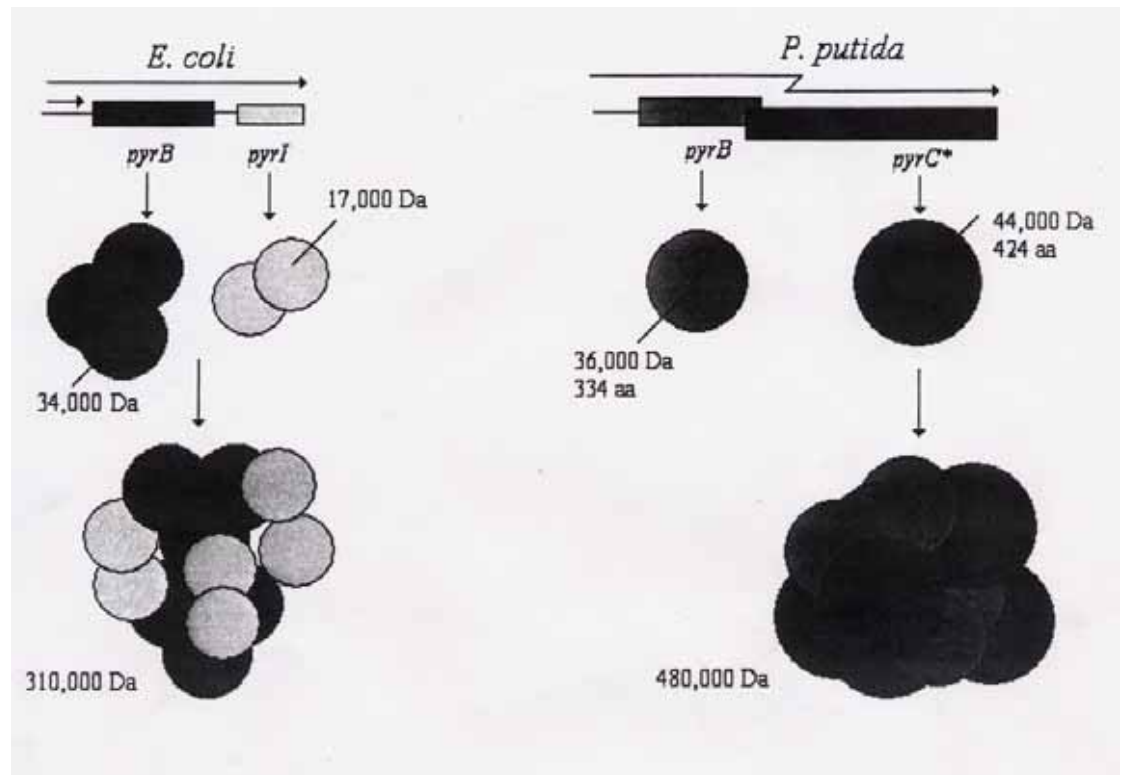


Figure 4. Synthesis of aspartate transcarbamoylase from *E. coli* and *Pseudomonas*. In *E. coli*, *pyrB* and *pyrI* are separated by 15bp while in *Pseudomonas* the *pyrB* and *pyrC'* genes overlap by 4bp. The *pyrB* gene encodes the catalytic ATCase in both cases while *pyrI* encodes a regulatory polypeptide in *E. coli* and *pyrC'* an inactive DHOase in *Pseudomonas* that is required for enzyme activity.

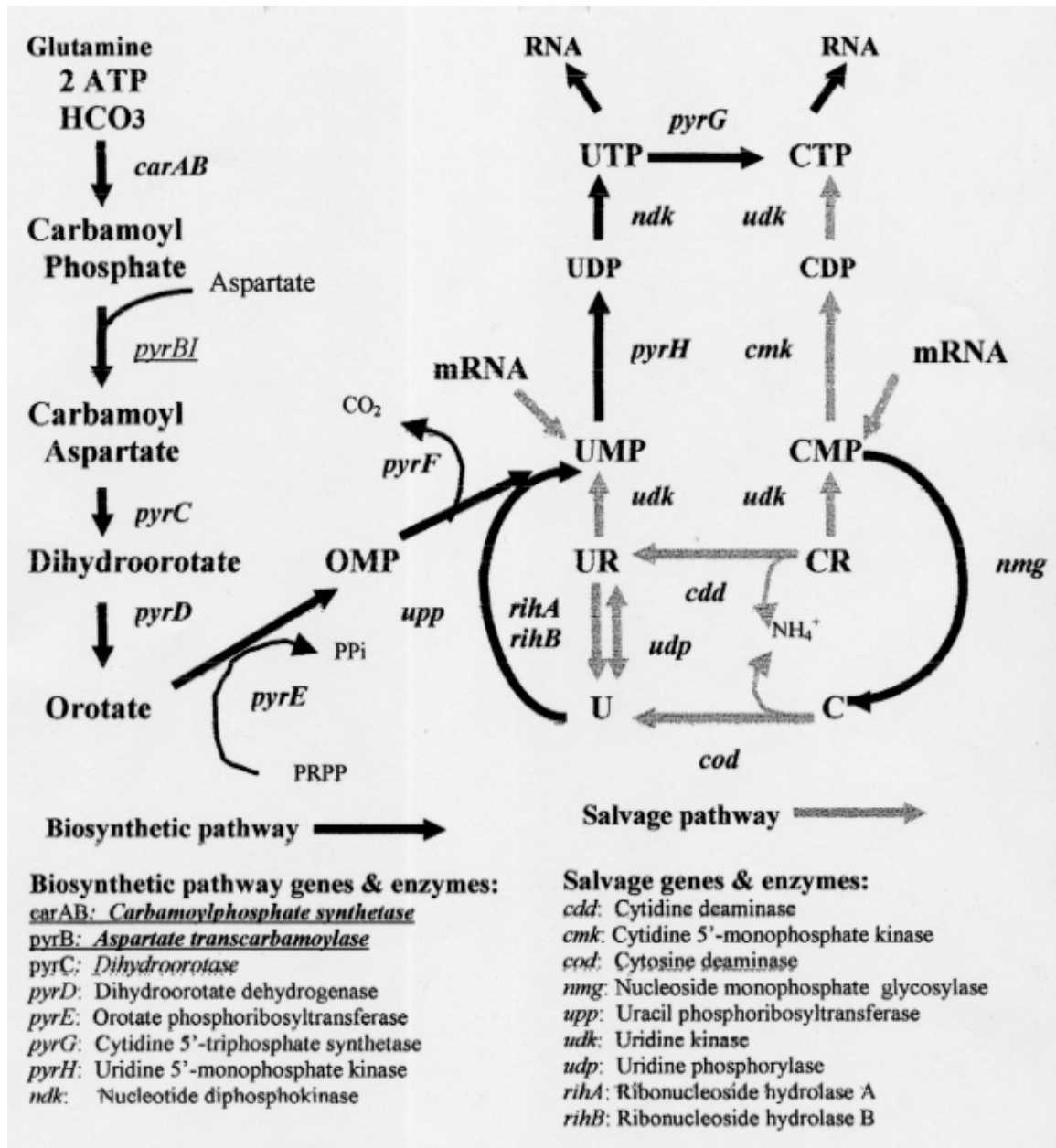


Figure 5. The pyrimidine biosynthetic and salvage pathways in *E. coli*.

In *E. coli*, the amino acid sequence of the two polypeptide chains is known. The catalytic region consists of two tryptophan residues besides a cysteine near the catalytic site of the enzyme (13). A similar site is found in the *Pseudomonas* enzyme (32).

Regulation of ATCase in *E. coli*

ATCase regulation in *E. coli* is by allosteric inhibition and activation. The enzyme is allosterically inhibited by CTP and activated by ATP. The binding of ATP and CTP to the regulatory sites was shown to be anti-cooperative, which means that they compete for a single site on the regulatory subunit (37). A primary- secondary effects model has been used to interpret the heterotropic interaction involved in the binding of these effectors to the enzyme. The nucleotides act by changing the affinity of the catalytic site for aspartate by local conformational changes. Therefore, this model reveals the fact that ATP reverses the effect of CTP and the opposite is also true (38). So, when ATP binds to the regulatory site of the enzyme, it promotes a local conformational change, which increases the affinity for aspartate to the catalytic site of the enzyme, this is called the primary effect. However, the secondary effect occurs in the presence of aspartate. The conformational changes that made by ATP increase the level of saturation of the catalytic sites by aspartate. These changes shift the T and R equilibrium towards the R state. On the other hand, CTP, when it binds to the regulatory site of the enzyme, promotes a local conformational change that decreases the affinity of aspartate for the catalytic site. Here, and in the presence of aspartate, these conformational changes decrease the saturation of the catalytic sites for this substrate and shift the T and R equilibrium toward the T form.

Class C enzymes are found in all Gram-positive bacteria. This class of ATCase is the smallest with a molecular mass of about 100kDa. Also, this class is insensitive to allosteric effectors. *Bacillus subtilis* ATCase is a trimer composed of three-33.5kDa polypeptides with a tertiary structure very similar to the *E. coli* catalytic subunits (36).

The class C ATCase consists of three catalytic polypeptide chains with a molecular mass of 102 kDa.

ATCase is found to exist in two different forms, which are called R and T states (Fig 6). A taut conformation (T-state) has a low affinity for aspartate, while the relaxed conformation (R-state) has a strong affinity for aspartate. The transitions from T state to R state involve the simultaneous breaking of interface localized bonds, and formation of the others at different interface sites (16, 18).

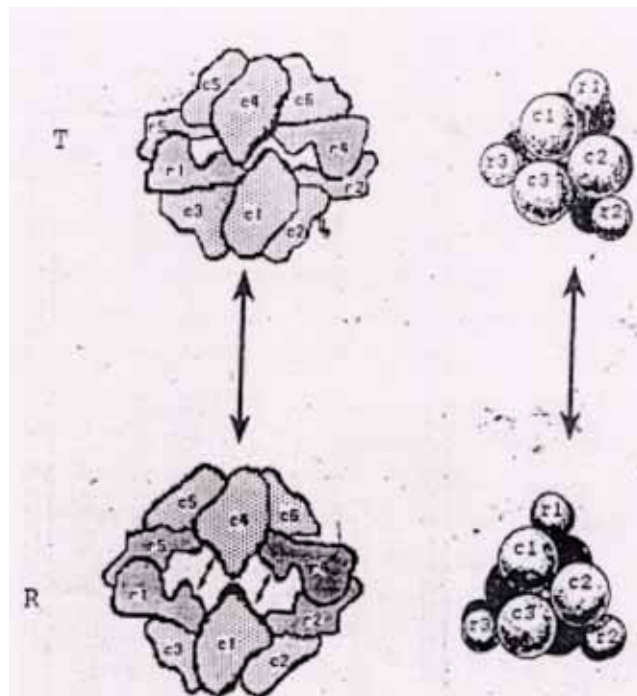


Figure 6. Schematic representation of the T to R transition of the *E. coli* ATCase. C = catalytic chain, r = regulatory chain.

In mammals, ATCase activity is associated with a single CAD polypeptide with three domains for CPSase, ATCase and DHOase, which catalyze the first three steps in pyrimidine biosynthesis (34). The mammalian ATCase domain is an unregulated trimer composed of 34kDa subunits (35). The similarity in sequence among the catalytic

polypeptides of *E. coli* ATCase, *B. subtilis* ATCase and eukaryotic ATCase domains suggests a highly conserved structure.

Protein Purification

Chromatography

Chromatography as well as all other protein purification methods takes advantage of relative differences between the chemical and physical properties of amino acids. Amino acids can be separated according to their solvent affinity or their electrical charge. In the partition method, the protein is allowed or forced to flow through a medium consisting of two phases-solid-liquid, liquid-liquid, and gas-liquid. In general due to the acid-base groups of amino acids, protein solubility depends on salts concentration, the polarity of the solvent, the pH, and the temperature.

The power of chromatography derives from the continuous nature of the separation process. At the end, the separated components are collected and submitted to further analysis. The various chromatography methods are classified according to their mobile and stationary phases. For example, in liquid-liquid chromatography there are immiscible liquids, one of which is bound to an inert solid support. Chromatographic methods are further classified according to the nature of the dominant interaction between the stationary phase and the substances being separated. For example, if the retarding force is ionic in character, the separation technique is referred to as ion chromatography, whereas if it is a result of adsorption of the solutes onto a solid stationary phase, it is known as adsorption chromatography.

A cell contains huge numbers of different categories of components, many of which closely resemble one another in their various properties. Therefore, the isolation

procedures for most biological substances use a number of independent chromatographic steps in order to purify the substance of interest according to several criteria. In this research, different chromatographic techniques are used in order to purify ATCase.

The first technique is Ion Exchange Chromatography. In this techniques, Ions that are electrostatically bound to an insoluble and chemically inert matrix are reversibly replaced by ions in solution (Fig 7). In the process, the solutes in the liquid phase, usually water, are passed through columns filled with a porous solid phase, usually a bed of synthetic resin particles, containing charged groups (Fig 8). Negatively charged resin attracts positively charged species whereas positively charged resin attracts negatively charged species (11).

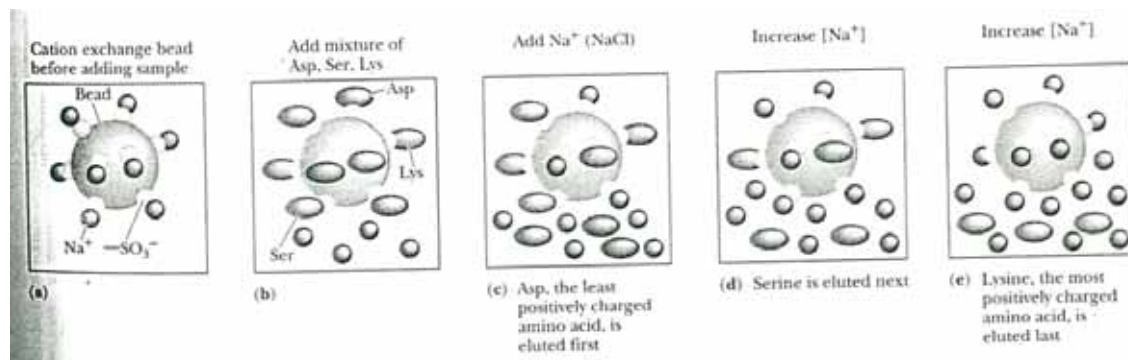


Figure 7. Mode of action of a cation exchange column.

The second chromatography method used in this research is Hydrophobic Exchange Chromatography. Sepharose is a bead-formed gel prepared from agarose. In its natural state agarose occurs as part of the complex mixture of charged and neutral polysaccharides referred to as agar. The agarose used to make Sepharose is obtained by a purification process, which removes the charged polysaccharides to give a gel with only a very small number of residual charged groups. Therefore, hydrophobic interaction

chromatography uses the hydrophobic nature of proteins in purifying them. Before running the chromatography, the column is prepared and regenerated as follows: the

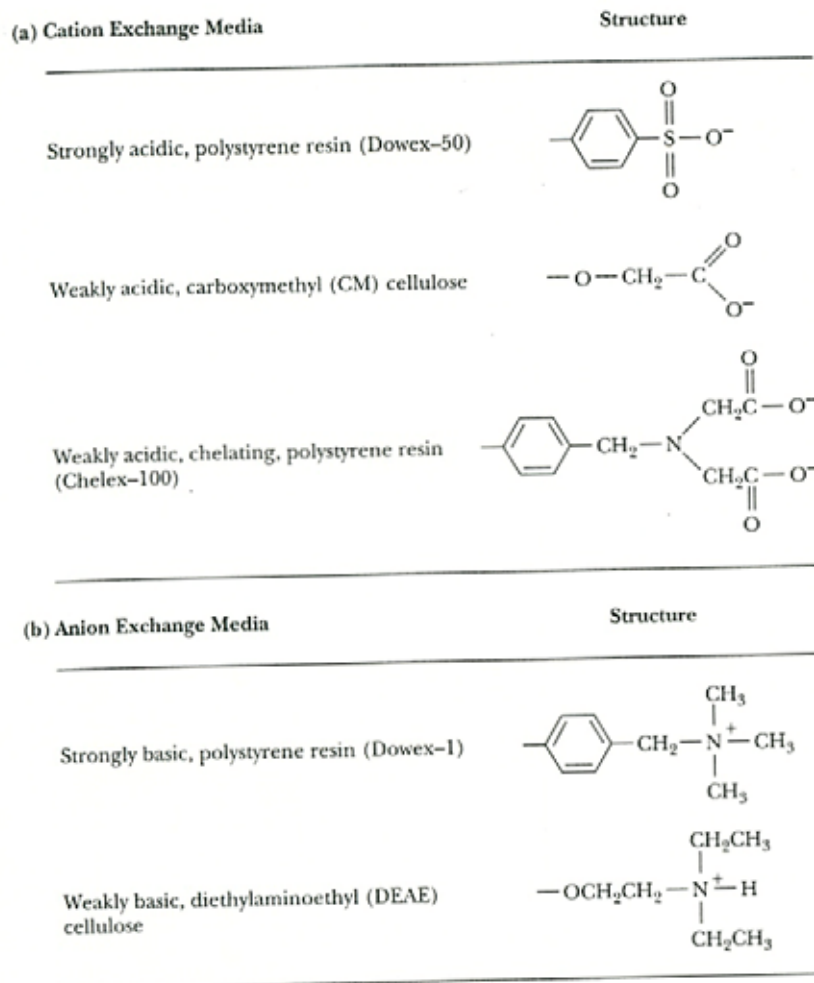


Figure 8. Cation and anion exchange resins commonly used for biochemical separation. agarose beads are washed twice with 50% ethanol, once with n-butanol and again twice with 50% ethanol and then with plenty of water. The column is then packed and equilibrated with high buffer. After regeneration of the column the ATCase is eluted.

Hydrophobic groups are covalently linked to the phenyl Sepharose matrix. Therefore, in the presence of high salt concentration, proteins bind to the phenyl groups by virtue of hydrophobic interaction. Proteins in a mixture can be differentially eluted

from the phenyl group by lowering the salt concentration or by adding solvent such as ethylene glycol to the elution fluid. Once the elution is finished the hydrophobic interaction chromatography gel can be reused several times depending on the quality of the buffer.

Enzyme Kinetics

The study of enzyme kinetics is very important in the field of molecular biology. From this study, the maximum reaction velocity that the enzyme attains can be determined. Its binding affinities for substrates and inhibitors can also be determined. Therefore, this information can be used to control and manipulate the course of metabolic events.

In 1913, Michaelis and Menten proposed a general theory of enzyme action. This theory was based on the assumption that the enzyme and its substrate associate reversibly to form an enzyme-substrate complex. There are some factors such as pH that can decrease or increase the enzyme activity. In general, enzymes are active only over a limited pH range and most have a particular pH at which their catalytic activity is optimal. The effects of pH on the enzyme activity may be due to effects on K_m or V_{max} or both. The other factor that can affect the enzyme activity is the temperature. In general, the rates of enzyme-catalyzed reaction are increased with increasing temperature. However, temperature above 50°C decreases the activity of most enzymes.

Enzyme Inhibition

Inhibitors of enzymes can be classified into two types. The first type is called reversible inhibitors. This type of inhibitor interacts with the enzyme in a noncovalent association. The other type is called irreversible inhibitors in contrast with the first type.

This type causes a stable and covalent alteration in the enzyme. The reversible inhibitors are of two kinds namely competitive and noncompetitive inhibitors. In the case of the competitive inhibitors, the substrate and the inhibitor compete for the same binding site on the enzyme, whereas the noncompetitive inhibitors are not binding to the same site as the substrate does.

The purpose of this study was to compare the effector response between the wild type *Pseudomonas putida* ATCase and a mutant ATCase lacking 11 amino acids at the N-terminus. The effectors used in this study were ATP, CTP, and UTP. After the protein (ATCase) was purified from both the wild type and the mutant type, the ATCase assay was done on each type. In this ATCase assay, ATP, CTP, and UTP were used as effectors since each had been shown to inhibit the enzyme by previous workers (32).

MATERIALS AND METHODS

Chemicals

Ampicillin monosodium salt, agarose, dibasic potassium phosphate, monobasic potassium phosphate, ammonium sulfate, sodium citrate, magnesium sulfate, thiamine, dextrose, Tris, chloride, ammonium persulfate, sodium dodecyl sulfate, and EDTA disodium salt were all provided by Fisher Scientific Co. Luria-Bertani medium, sodium chloride, potassium chloride, bromophenol blue, and granulated agar were provided by Difco laboratories. Aspartate, carbamylphosphate, isopropyl-B-thio-galactopyranoside (IPTG), bisacrylamide, acrylamide, and calcium chloride dihydrate were provided by Sigma Chemical Company. Glycerol and ethanol were provided by EM Science. Type II H₂O was generated by a Barnstead NANOpure ultrapure water system. All type II H₂O was then autoclaved. Also, different chemical materials have been used. Antipyrine and monoxime are required for ATCase assay. Effectors, ATP, CTP, and UTP were used in this study at final concentration of 2mM.

Media and Bacterial Strains

Luria-Bertani (LB) enriched medium (3) was used for strains and plasmid constructions with the following antibiotics as required : ampicillin, 100 µg/mL (*E. coli*); kanamycin, 50 µg/mL (*E. coli*).

The *E. coli* minimal medium (25) contained the following (grams per liter of distilled/deionized water): K₂HPO₄, 10.5; KH₂PO₄, 4.5; (NH₄)₂SO₄, 1.0; Na₃ citrate, 0.5. This solution was autoclaved and cooled to 50°C before adding glucose (10mL of 20% solution), MgSO₄ (1mL of 1M), thiamine (5mL of 0.4% solution) and arginine

(50µg/mL). The glucose was used as a carbon source. All *E. coli* strains were grown at 37°C. All strains and plasmids used in this study are listed in Table1 .

Preparation of Competent Cells for Transformation

A single colony was inoculated into 50 mL of L-Broth and incubated, with shaking at 37°C until OD₆₀₀ reached between 0.2 and 0.4 OD units. The cells were then harvested by centrifugation at 1300 xg for 15 min. Then the pellet was resuspended in 20mL of cold 0.1 M CaCl₂ and then incubated at 0°C for 20 min, and harvested by centrifugation at 1300 xg for 15 min. Next, CaCl₂ was used to resuspend the pellet. After 24 hours, 50% glycerol was added to the cells giving a final concentration of 15% glycerol, which were rendered competent.

Genetic Manipulation

Transformation of DNA in *E. coli* TB2 was carried out by the following method (14):

3µL (600ng) from each plasmid used in the transformation was added to a 1.5mL microfuge tube. Then 200µL from the competent cell was added to the same tube, and placed on ice for 10 min. Then it was incubated in water bath (42°C) for 2 min. 1mL of LB broth medium was added to the tube and incubated for 45min in gyratory shaker at 37°C and 250 rpm. This incubation allows the cells to recover. Finally the transformed cells were spread onto plates of LB agar supplemented with ampicillin and kanamycin and then were grown on *E. coli* minimal medium plates with arginine , ampicillin and kanamycin and the growth was monitored.

In this study two transformations were done. The first one included pKPB-11 (– 11aa PyrB) (Fig 9) and pDBPC' (PyrC') (Fig 10), this is for the mutant type (Fig 12).

The second transformation included pAKB10 (PyrB WT) (Fig 11) and pDBPC' (PyrC'), and this is for the wild type (Fig 12).

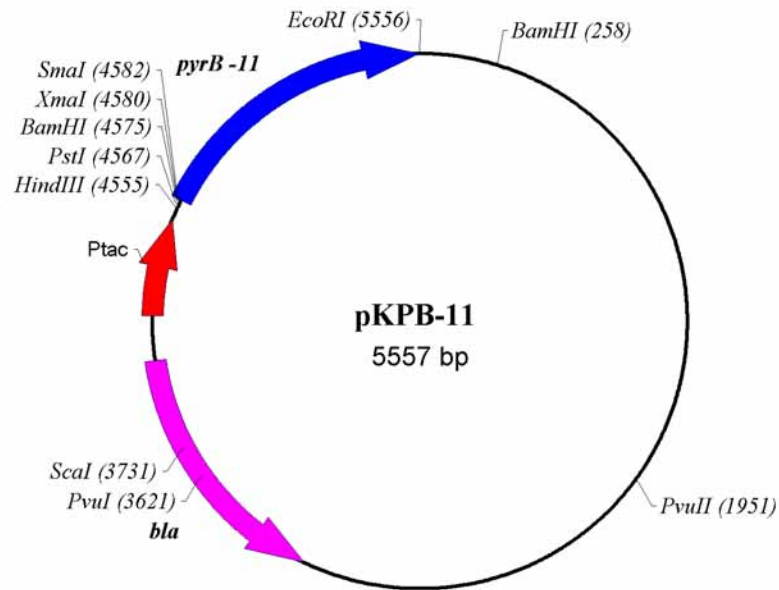


Figure 9. *P. putida pyrB-11* insert in the expression vector pKK223-3.

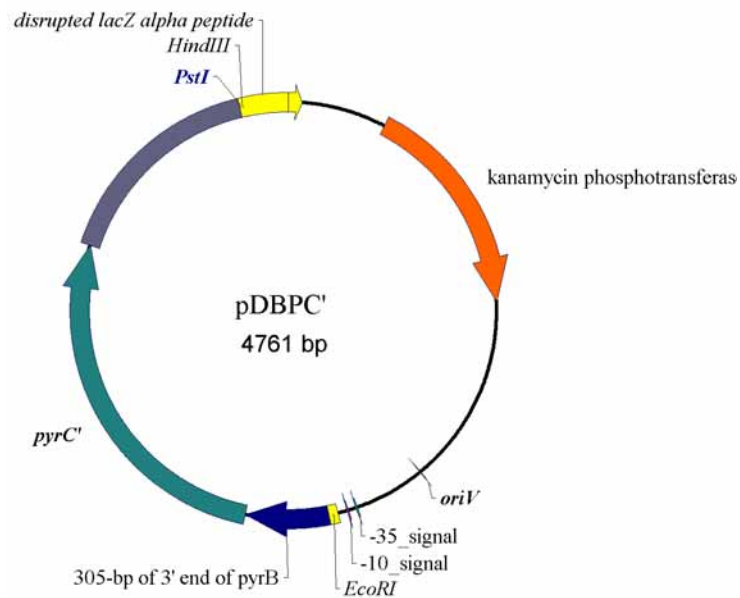


Figure 10. *P. putida pyrC''* in pK184.

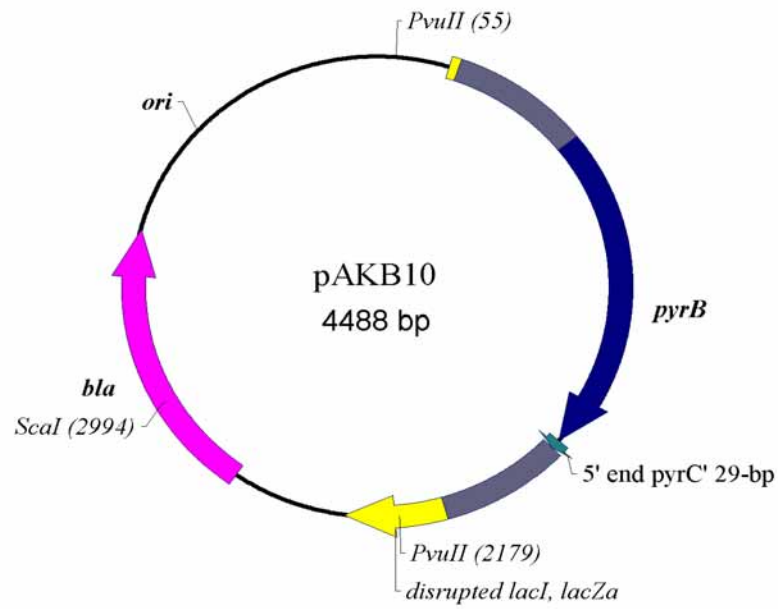


Figure 11. *P. putida* *pyrB* in pUC19.

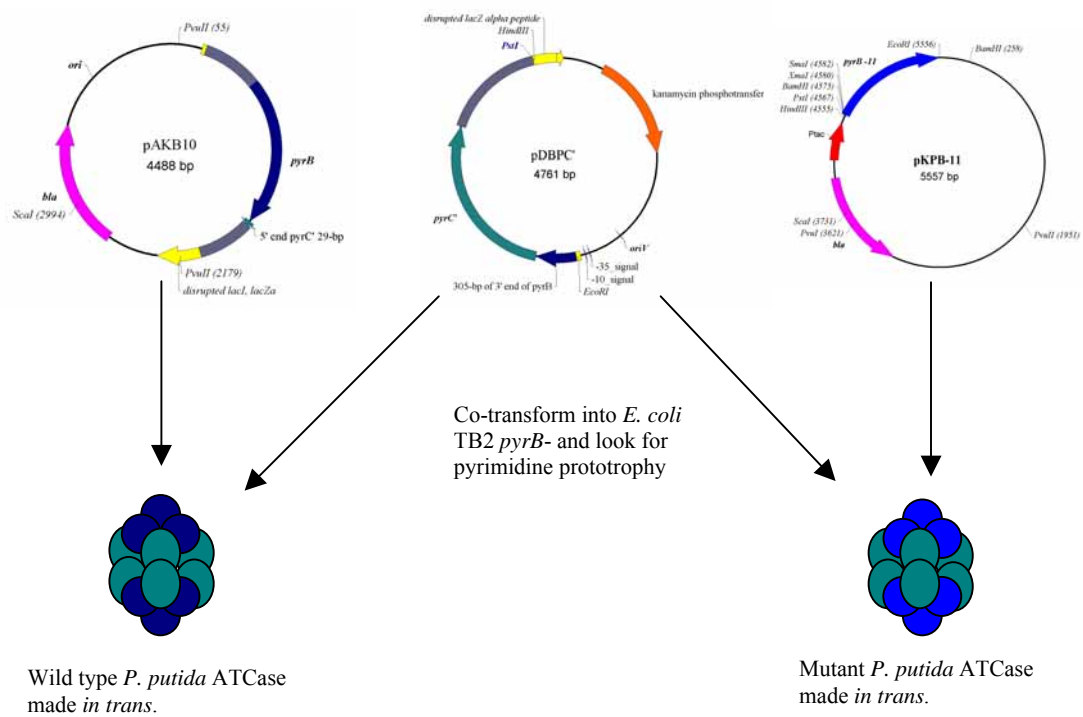


Figure 12. *P. putida* ATCase made in trans from both wild and mutant types.

From the plates that have the transformed cells, many colonies were inoculated in *E.coli* minimal medium plates using toothpick process. Those plates were supplied with the chemicals they need and incubated at 30°C overnight. Then one colony was inoculated in 50mL *E. coli* minimal medium.

For 50mL of *E.coli* minimal medium, 500μL of 20% glucose, 250μL of 0.4% thiamine, 50μL of 1.0M of MgSO₄, 250μL of 2.54% arginine, 50μL of Amp₁₀₀, and 50μL of Kan₅₀ were added. This 50mL was incubated at 30°C and the growth noted; 10mL from this culture was inoculated in one liter of *E. coli* minimal medium (4L from the medium was used). These cultures were incubated at 30°C for about 16 hours.

Purification of *P. putida* Wild Type ATCase and Mutant ATCase

The *P. putida* ATCase was purified in *E. coli* TB2. The enzyme was purified from 4 liters of culture by the method of Adair and Jones (1).

The cells were incubated at 30°C for about 16 hours. Then, the cells were broken by using the French pressure cell (French Press). This machine achieves cell lysis by placing the sample under high pressure followed by a sudden release to atmospheric pressure. The rapid change in pressure causes cells to burst. This procedure works very well for moderate volumes (10-30mL), but becomes technically difficult with smaller volumes . After breakage, the cells were resuspended in ATCase buffer. The broken cells were transferred to a centrifuge tube (using SA600), and the centrifugation was done at 10700 xg for 1 hour. This centrifugation precipitates the cell wall and the cell membrane fractions. Then 2% streptomycin cut was done on the supernatant. The solution was centrifuged at 10700 xg for 30 min. A 30% ammonium sulphate cut was carried out after this centrifugation. Then a 50% ammonium sulphate cut was completed. The

centrifugation done at 10700 xg for 30 min as before. The pellet was resuspended in 1mL ATCase buffer.

Dialysis

Dialysis is a method for concentrating protein solution by dialyzing against a hygroscopic environment. The protocol for this process is as following:

- 1- Make two tight knots at one of the tubing.
- 2- Using a pipet or funnel, deliver a protein solution into the dialysis tubing.
- 3- Tie a double knot at the end of the tubing and place the closed dialysis bag in >10 volume of dialysis buffer. The buffer should be stirred gently with a magnetic stir bar to improve solute exchange. Equilibrium occurs after several hours of dialysis, and the dialysis buffer may have to be changed several times until certain buffer components are sufficiently diluted.

An ionic exchange column was used with DEAE Sepharose as a matrix. Then, all other ATCasees were purified using a phenyl sepharose Hydrophobic Interaction Column (HIC) following ammonium sulphate precipitation and the proteins were eluted with a predetermined salt concentration. Sepharose is a bead-formed gel prepared from agarose. Agarose, in its natural state, occurs as part of the complex mixture of charged and neutral polysaccharides referred to as agar. Hydrophobic interaction chromatography uses the hydrophobic nature of proteins in purifying them. Before running the chromatography, the column is packed and equilibrated with high buffer. Here, hydrophobic groups are covalently linked to the phenyl sepharose matrix. Therefore, in the presence of high salt concentration of ammonium sulphate, proteins bind to the phenyl groups by virtue of hydrophobic interaction. ATCase, the subject of this study, was eluted when the salt

concentration was at 0M, which is the lowest concentration. In other words, it is eluted with the ATCase buffer. Then the ATCase assay was performed on purified proteins. Protein concentration was determined by the method of Bradford (6).

Aspartate Transcarbamoylase Assays

The ATCase assay was the same for both the wild type and the mutant type and is routinely carried out in our lab. The following reagent were mixed in the following amounts: 54 μ L ddH₂O, 28 μ L tri-buffer (50mM MES, 100mM diethanolamine, and 51mM N-ethylmorpholine, pH 9.5), and 35 μ L 20X aspartate (200mM aspartate). 87 μ L of this mixture was distributed into the microtiter wells. Then, to these wells 3 μ L from the enzyme was added. The reaction was initiated by adding 10 μ L of carbamoylphosphate to each well. The samples were incubated 15 minutes at 30°C. After incubating, the reaction was stopped by adding 100 μ L of stop mix reagent (2 parts antipyrine and 1 part monoxime, combined just before use). The samples were then incubated in the presence of light for about 2 hours at 60°C. the samples were then read on the Molecular Devices Company microplate reader. The ATCase was assayed under two sets of conditions, under varying aspartate concentration and under varying carbamoylphosphate concentration.

Aspartate concentrations ranged from 0.1mM aspartate to 20mM aspartate while the carbamoylphosphate concentrations ranged from 0.1mM CP to 3mM CP.

The ATCase activity was assayed by measuring CAA formation at 30°C with modifications using the color development procedure (12, 29). The nucleotide effectors that have been used in this study include: 20mM ATP, 20mM CTP, and 20mM UTP. In the microtiter wells, the first set was run without any effector, the second set included

ATP, the third included CTP, and the last one included UTP. The amount that has been added from the effectors (10 μ l) was adjusted with H₂O.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This method separates proteins based primarily on their molecular weights. SDS binds along the polypeptide chain, and the length of the reduced SDS-protein complex is proportional to its molecular weight. The separating gel was prepared by degassing a mixture containing 5mL of 30% acrylamide/ 0.8% bisacrylamide, 2.5mL of 4X Tris-HCl/SDS pH 8.8, then, 0.02g of 10% ammonium persulfate was added. This solution was then mixed with 5 μ L of TEMED. Once the TEMED was added to the mixture, the separating gel was added to the apparatus with a Pasteur pipet to a height of 11 cm.

Another Pasteur pipet was used to cover the top of the gel with a 1cm layer of H₂O-saturated isobutyl alcohol. The gel was allowed to polymerize for one hour at room temperature. While the gel was polymerizing, a stacking gel was made by combining 0.67mL of 30% acrylamide/0.8% bisacrylamide, 1mL of 4X Tris-HCl pH 6.8, and 2.3mL ddH₂O. the mixture was degassed for 15 minutes and 0.02g of 10% ammonium persulfate and 5 μ L of TEMED were added. Then, the solution was mixed by swirling. The alcohol was then poured off of the gel. The stacking gel solution was then added and a comb inserted. The gel was allowed to polymerize for 45 minutes.

After polymerization, the samples were heated for about 3 minutes at 100°C in a sealed microcentrifuge tube. The comb was then removed from the gel and the wells were rinsed with 1X electrophoresis buffer. The samples were then loaded into the wells, as were the standards. 1X electrophoresis buffer was then used to fill in all the empty wells and to fill the chamber to the upper electrode. Then, the chamber was sealed and

connected to the power supply and run at 100 volts until the bromophenol blue tracking dye reached the bottom of the separating gel (approximately 1 hour). The gel was then stained with coomassie blue for about 24 hours.

Nondenaturing Gel Electrophoresis (Native Gel Electrophoresis)

This gel separates proteins based on their size and charge properties. This method is able to separate molecules which differ by a single unit charge. The separating gel was prepared by mixing 3.3mL of 30% acrylamide/0.8% bis-acrylamide, 2.5mL of 4X Tris-Cl pH 8.8, and 4.2mL ddH₂O. The mixture was then mixed with 0.02g of 10% ammonium persulfate and 5μL of TEMED. Directly after the TEMED was added, the separating gel solution was added to the apparatus with a Pasteur pipet. A 1 cm gap was left at the top of the gel plates for pouring the stacking gel later. Then, another pipet was used to cover the top of the gel with a 1cm of isobutyl alcohol. Gel was allowed to polymerize for about 45 minutes at room temperature. Then, the stacking gel was made by combining 0.67mL of 30% acrylamide/0.8% bis-acrylamide, 1mL of 0.5M Tris pH 6.8, and 2.3mL of ddH₂O. then, 0.02g of 10% ammonium persulfate and 5μL of TEMED were added to the mixture. The alcohol was then removed and the stacking gel solution was added and the comb was inserted. The gel was allowed to polymerize for about 45 minutes. The comb was then removed from the gel and the wells were cleaned. The samples were then loaded into the wells. 1X electrophoresis buffer was then used to fill the chamber to the upper electrode. The buffer contained 3.02 g of Tris, 14.4 g of glycine in 1 liter of ddH₂O at pH 8.3 was used as the electrophoresis buffer. The chamber was sealed and connected to the power supply and electrophoresed at 100 volts until the dye reached the bottom of the separating gel. The gel was then stained to show only ATCase activity.

The ATCase activity can be detected directly on the gel by activity staining, a method developed by Bothwell (5) and further modified by Kedzie (19). The gel was placed in ice-cold 50mM histidine (free base), pH7.0, for 5 minutes to which 5mL of 1.0M aspartate and 10mL of 0.1M carbamoylphosphate were added and allowed to react for 20 minutes. The gel was rinsed three times with ice-cold distilled water to remove reactants. Each wash should be done for 5-10 minutes in order to avoid a dark background after finishing the staining process. The enzymatically released P_i trapped in the gel was precipitated by a reaction with 3 mM lead nitrate in ice-cold 50 mM histidine, pH 7.0, for 10 minutes. The soluble lead ions were removed with three changes of ice-cold water. If the protein samples contained ATCase, the ATCase band can be visualized as the white band after this step. The gel was then stained with 1% sodium sulfide, which converted the white lead phosphate precipitate into dark lead sulfide bands.

Table 1. Strains and Plasmids

<i>Strain(*) or Plasmid (*)</i>	<i>Description or Relevant Phenotype</i>	<i>Source</i>
TB2* (<i>E. coli</i>)	Δ <i>pyrBI</i> , <i>argI</i> , <i>argF</i>	W.D. Roof
PKPB-11 [‡]	-11aa <i>PyrB</i> (<i>P. putida</i>) in pKK223-3	A. P. Kumar
pK184 [‡]	p15a replicon, Kan ^r	New England Biolabs
pAKB10 [‡]	<i>pyrB</i> (<i>P. putida</i>) in pUC19	A. P. Kumar
pDBPC ^{’,‡}	<i>P. putida pyrC’</i> in pK184	D. M. Brichta

RESULTS

Transformation of Plasmid pAKB10 (*P. putida pyrB* gene) and pDBPC' (*P. putida pyrC'* gene) into *E. coli* TB2

E. coli TB2 strain was used in the transformation in this study. This strain is devoid of ATCase activity due to deletion of the *pyrBI* region. When the *E. coli* TB2 strain was co-transformed with both plasmids, one carrying a functional *pyrB* and the other carrying *pyrC'*, the strain grew in the absence of uracil. The transformants were first plated on LB agar containing ampicillin and kanamycin. Then the transformants were grown in *E. coli* minimal medium with glucose as a carbon source as described in Materials and Methods. If the transformed plasmids encoded an active ATCase, then these transformed cells should grow in the absence of uracil. Because the transformed cells grew in the *E. coli* minimal medium, then an active ATCase was encoded and complemented the *E. coli* TB2 strain. This ATCase was purified (as described in Materials and Methods) and used for further study.

Transformation of Plasmids pKPB-11 (*P. putida* –11 aa *pyrB* gene) and pDBPC' (*P. putida pyrC'* gene) in *E. coli* TB2

In contrast to the first transformation, here the mutant *pyrB* was used, which has 11 amino acids deleted at the N-terminus. When the *E. coli* TB2 strain was transformed with both plasmids (pKPB-11 and pDBPC'), the strain grew in the absence of uracil. As above, the transformants were plated on LB agar containing ampicillin and kanamycin. Then, the transformants were again grown in *E. coli* minimal medium with glucose as a carbon source and supplemented with arginine. These transformants grew in

the absence of uracil. Thus, the plasmids encoded an active ATCase that complemented the *pyrB* mutant. This ATCase was then purified and used for further study.

Purification of *P. putida* ATCase from Wild Type and the 11aa Deletion Mutant

Both the wild type and the mutant ATCases were purified from *E. coli* TB2. The cells were collected and resuspended in breacking buffer. This cell suspension was passed twice through a French press at 1260 pounds per square inch. after breaking the cells, 30% and 50% ammonium sulfate cuts were done. After 50% cut, the pellet was resuspended in ATCase buffer and dialyzed overnight. Then ATCases were purified using a phenyl sepharose hydrophobic interaction column (HIC) following ammonium sulphate precipitation and the protein were eluted with a predetermined salt concentration. When ATCase wild type was purified, SDS- polyacrylamide gel electrophoresis was run to show the purity of the enzyme. Results (Fig 13) showed that ATCase was partially pure.

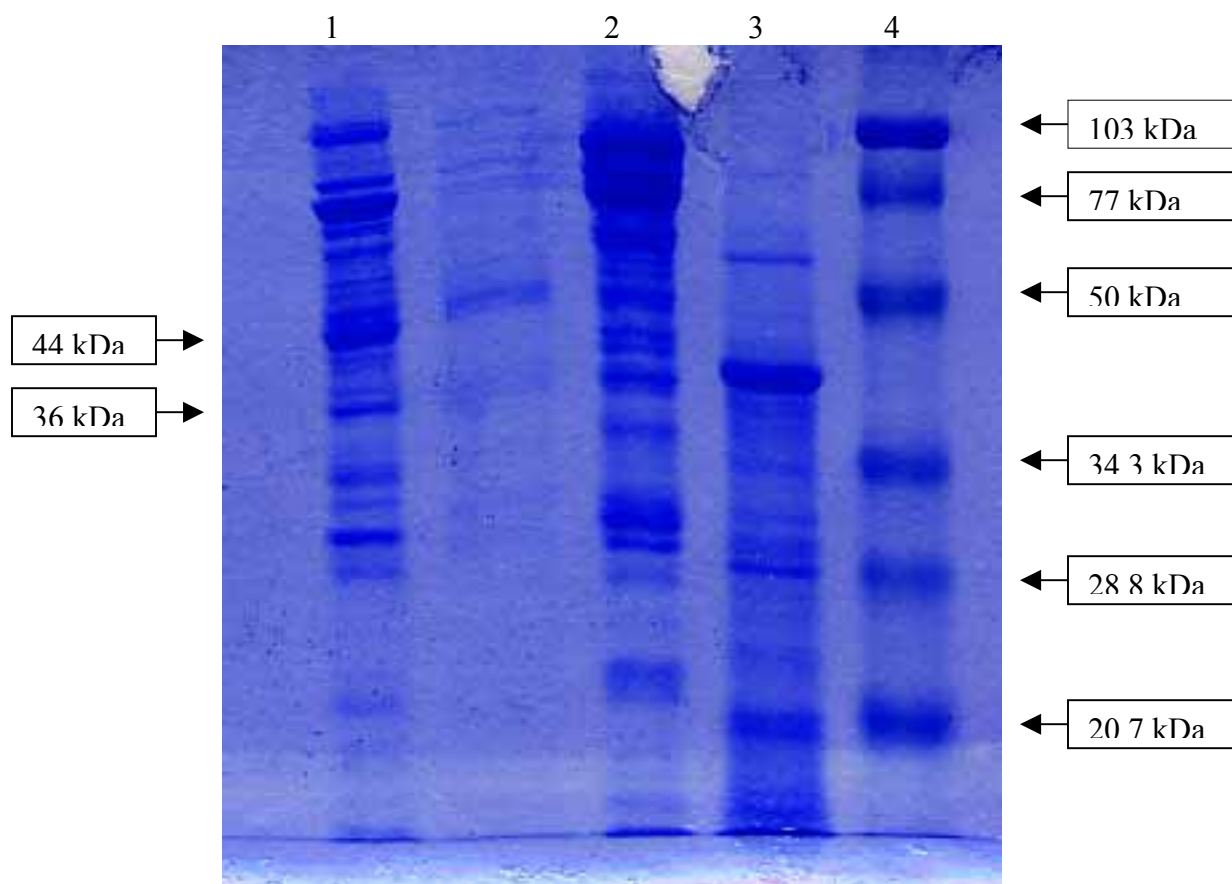


Figure 13. 12% SDS denaturing polyacrylamide gel and coomassie stained -11 aa *P. putida* ATCase (lane 1), wild type *P. putida* ATCase (lane 2), *E. coli* (lane 3), and the marker (lane 4).

Native gel electrophoresis was run to estimate the size of the enzyme. The results from this gel are shown in Fig 14. The wild type ATCase has a molecular mass of 484kDa. The mutant ATCase also showed a molecular mass of 484kDa.

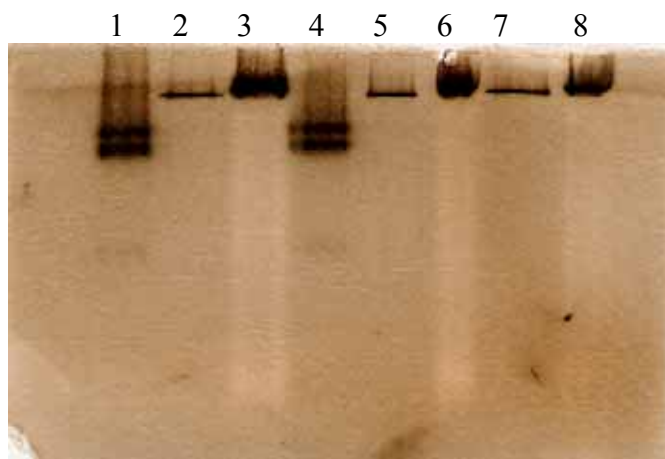


Figure 14. 10% non-denaturing gel stained for ATCase activity. (1 - *E. coli* ATCase; 2 - *P. putida* wild type ATCase; 3 - *P. putida* mutant; 4, 5, 6 - same order but exposed to 42°C; 7 - *P. putida* wild type exposed to 65°C; 8 - *P. putida* mutant ATCase exposed to 65°C.

Similarly, when the ATCase mutant was purified, it was run in SDS-polyacrylamide gel electrophoresis and in native gel electrophoresis to show both the purity and the size of the enzyme. However, the results indicated that the ATCase mutant type was also partially pure and these results are shown in Fig 13. From this figure, the two polypeptides with molecular masses of 36 kDa (PyrB) and 44 kDa (PyrC') were seen in both the wild type and the mutant type. The results from the native gel are shown in Fig 14. This figure showed that the active dodecamer was assembled in both the wild type and the mutant.

ATCase Assay

ATCase assay was performed on the partially purified ATCase from the deletion mutant and on the partially purified wild type ATCase. Kinetic studies were then performed. The following nucleotides were used to show effector response: ATP, CTP, and UTP at final concentrations of 2mM for each.

Aspartate saturation curves and carbamoylphosphate saturation curves were determined for both the deletion mutant ATCase and for the wild type ATCase. For the wild type, the aspartate saturation curves are shown in Fig 15. From this figure, it is clear that the specific activity is decreased when the nucleotides (ATP, CTP, and UTP) were added with ATP being the most effective. These results indicate that all nucleotides inhibit the activity of the enzyme.

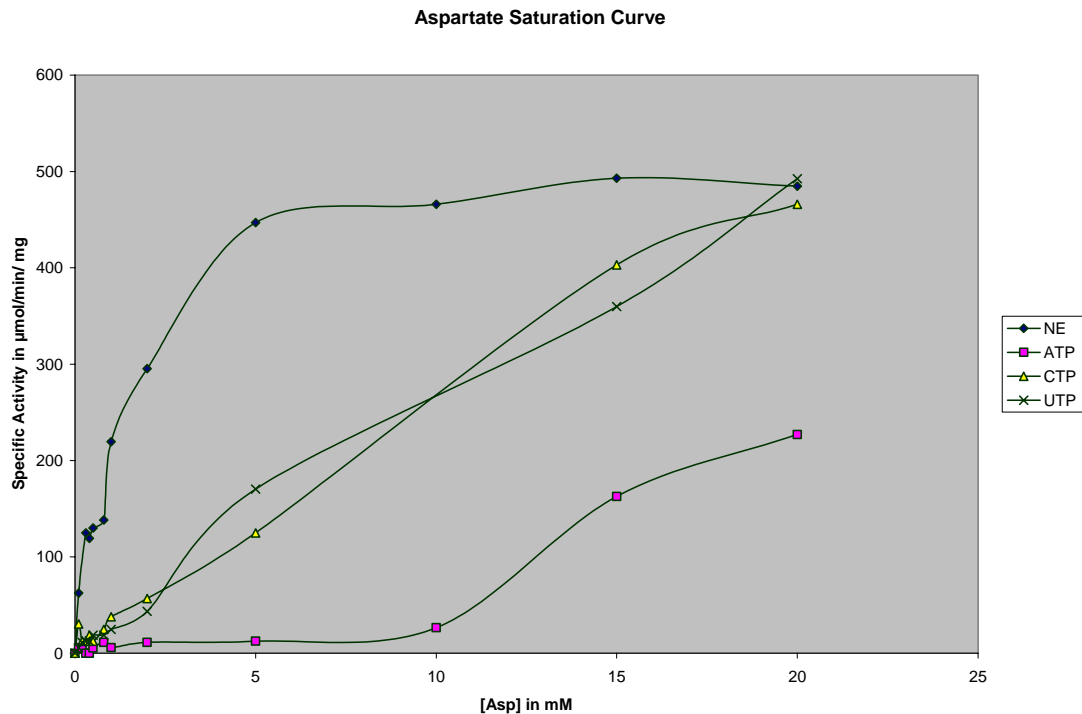


Figure 15. Aspartate saturation curves of partially purified wild type ATCase from *P. putida* expressed in *E. coli* TB2. At concentration of 5mM of aspartate, ATP shows 98% inhibition, UTP 64% and CTP 64%.

Carbamoylphosphate saturation curves are shown in Fig 16. From this figure, it is clear that the nucleotide ATP does not inhibit the wild type ATCase but both UTP and CTP activate the enzyme. This suggests that ATP does not act as an inhibitor of carbamoylphosphate binding, while an increase in specific activity was observed when the effector molecules CTP and UTP were added. In this case, CTP and UTP seem to

activate the enzyme. The final concentration of aspartate in this carbamoylphosphate saturation curve was 20mM. However, when the final concentration of the aspartate was held constant at 1.5mM, which is the K_m for aspartate, the curves suggested that all of the nucleotides (ATP, CTP, and UTP) inhibited the activity of the enzyme more than 90% (Fig 17).

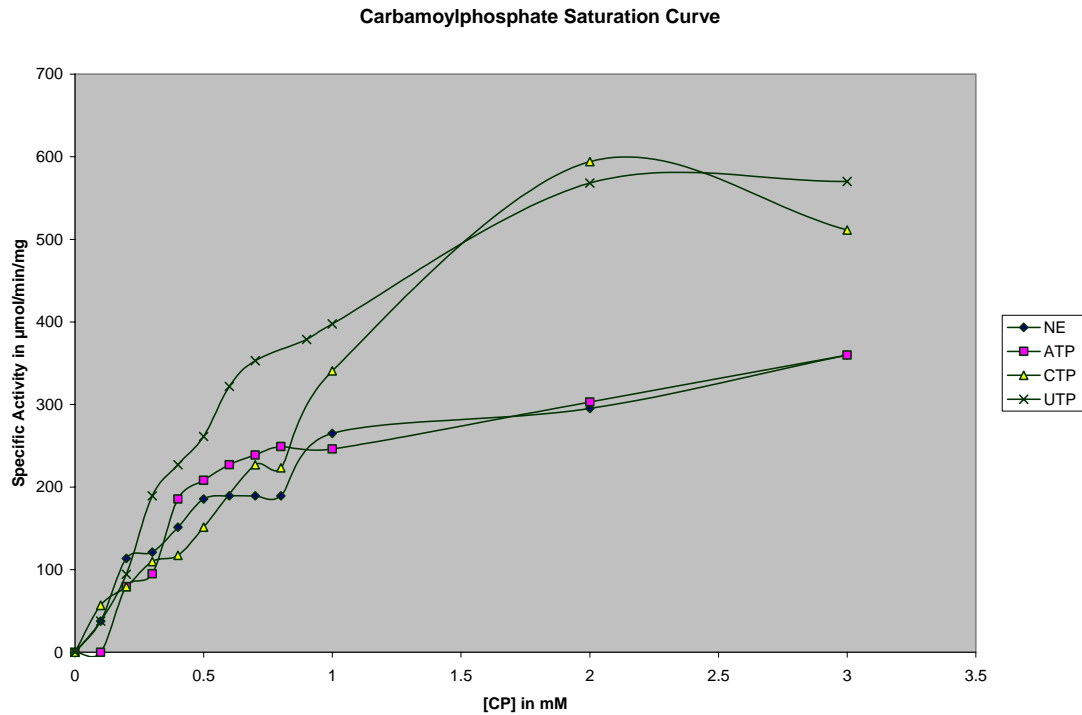


Figure 16. Carbamoylphosphate saturation curves of partially purified wild type ATCase from *P. putida* expressed in *E. coli* TB2. Carbamoylphosphate concentration was varied while aspartate was held constant at 20mM.

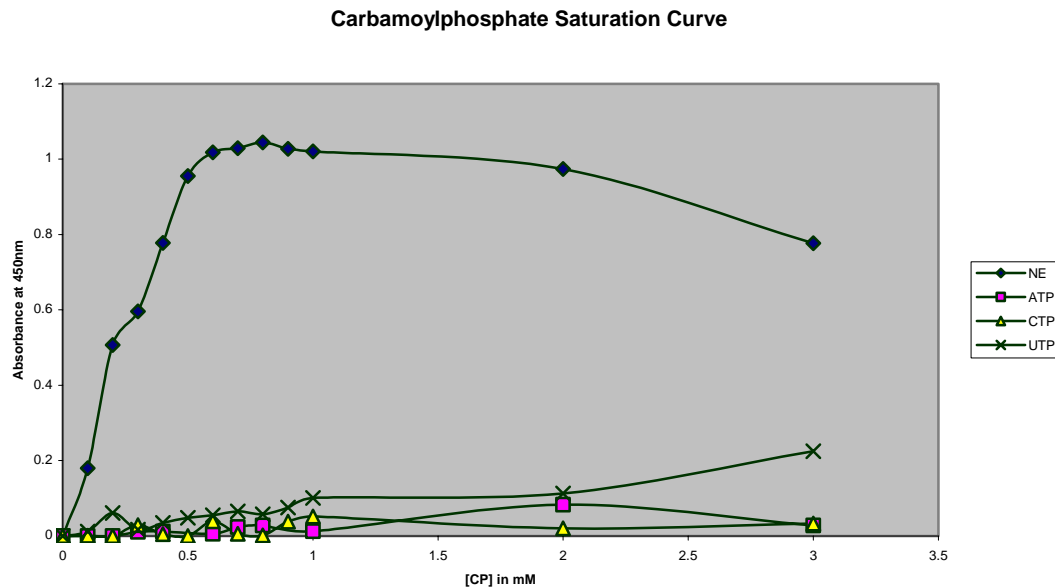


Figure 17. Carbamoylphosphate saturation curves of partially purified wild type ATCase from *P. putida* expressed in *E. coli* TB2. Carbamoylphosphate concentration was varied while aspartate concentration was held constant at 1.5mM, the K_m for aspartate. All of the effectors show an inhibition more than 90% in this case.

The same assay was done on the purified deletion mutant ATCase. In kinetic studies the same effector molecules were used (ATP, CTP, and UTP). Aspartate saturation curves for this mutant type are shown in Fig 18. Results from these assays indicated that CTP showed no effect on the enzyme activity until 20mM aspartate reached which seems to indicate substrate inhibition. Whereas, ATP and UTP inhibited the activity of the enzyme, which indicated that the binding site for CTP had been deleted or altered due to the deletion of the first 11 amino acids at the N-terminus of the PyrB polypeptide.

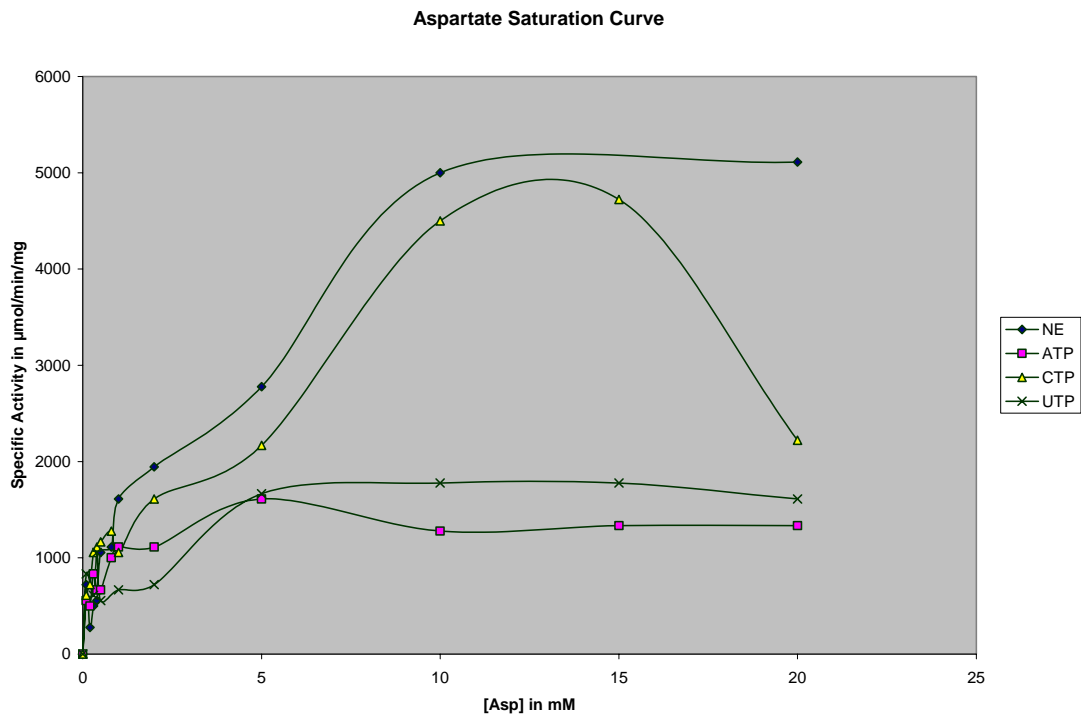


Figure 18. Aspartate saturation curves of partially purified mutant ATCase from *P. putida* expressed in *E. coli* TB2. Aspartate concentration was varied while carbamoylphosphate was held constant at 5mM AT [Asp] K_m , ATP shows 40% inhibition, UTP 70%,and CTP 10%.

Carbamoylphosphate saturation curves determined for the mutant ATCase are shown in Fig 19. In these curves CTP showed little or no effect on the enzyme activity, while ATP and UTP gave significant inhibition.

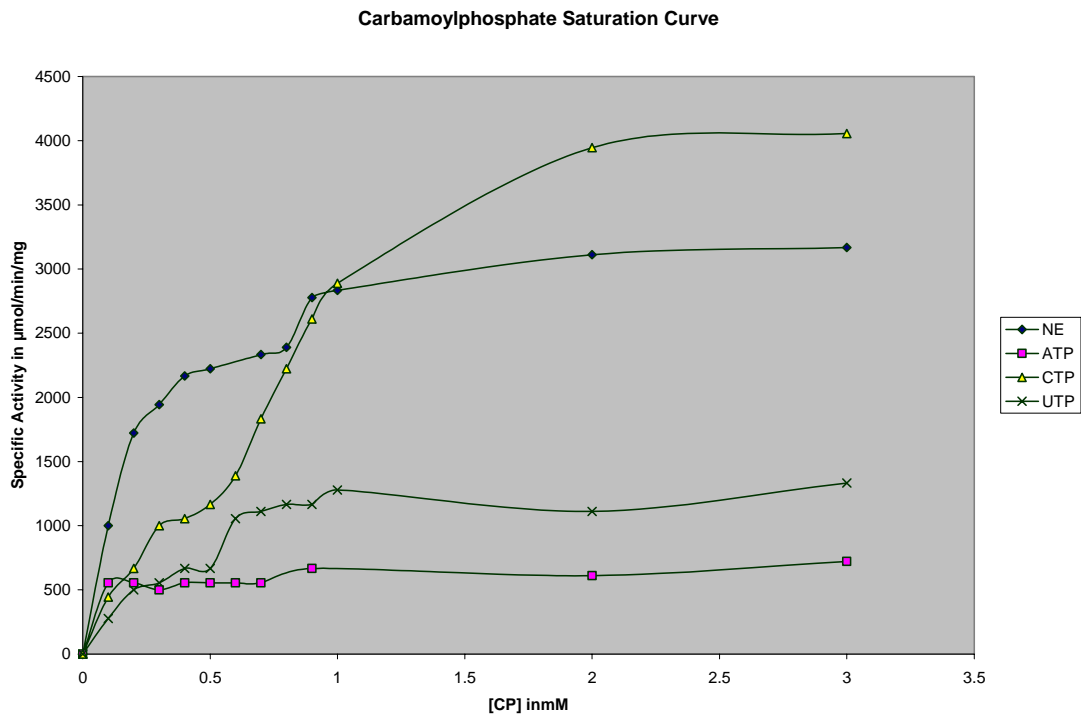


Figure 19. Carbamoylphosphate saturation curves of partially purified mutant ATCase from *P. putida* expressed in *E. coli* TB2. Carbamoylphosphate concentration was varied while aspartate was held constant at 20mM. At 1mM of [CP], ATP shows 83%, UTP 58%, while CTP shows 0% or no inhibition.

DISCUSSION

Pseudomonas ATCases, which represent class A enzymes, have been the subject of numerous studies (8, 15, 32, 40). ATCases from a number of *Pseudomonas* species have been purified and characterized (1, 14, 30, 32). Class A ATCase is more widely distributed in the bacterial world than is the *E. coli* ATCase. The *P. putida* ATCase, the subject of this study, has an amino terminal carbamoylphosphate-binding domain like the *E. coli* ATCase and a carboxy- terminal aspartate domain.. ATCase in *E. coli* is active only after assembly into a trimer since the active site is formed from two discrete polypeptides half-sites (30). These are therefore three active sites per trimer.

Sequence analysis of the cloned *P. putida* ATCase genes showed that ATCase was expressed from two ORFs that overlap by 4bp. The first ORF is *pyrB*, which encodes a 36.4kDa polypeptide. The second ORF is a *pyrC'*, which encodes a 44.2-kDa polypeptide (33). The *pyrC'* gene product is needed for the ATCase activity, and it has been shown that the active *pyrC* gene product from elsewhere in the chromosome could not substitute for the function of the *pyrC'* gene product (33).

E. coli TB2 strain, which is devoid of ATCase activity due to deletion of the *pyrBI* region, was transformed with a plasmid carrying the *P. putida pyrB* gene and another plasmid carrying the *pyrC'* gene. This transformation results in the assembly of the dodecameric holoenzyme. However, if extracts containing the expressed PyrB polypeptides were mixed *in vitro* with extracts containing the expressed PyrC' polypeptides, an active dodecamer was not assembled (30). On the other hand, an active dodecamer was only made if the mixed extracts were denatured completely and then allowed to renature slowly (30). In contrast, the *E. coli* PyrB- and PyrI containing

extracts could be merely mixed and an active holoenzyme assembled without any treatment. However, this would suggest that the *Pseudomonas* enzyme is assembled differently from the *E. coli* enzyme. When *E. coli* TB2 strain was transformed with a plasmid carrying –11 aa *pyrB* gene and another plasmid carrying *pyrC*' gene, an active ATCase was assembled and this ATCase represents the mutant type in this study.

In a separate study, it has been shown that *Pseudomonas* ATCase has a functional nucleotide effector binding site which resides at the N-terminus (within the first 60 amino acids) of the *PyrB* catalytic polypeptide (33). In the present work, kinetic studies have been done on both the wild type ATCase and the mutant ATCase. The changes made at the N-terminal region of the *PyrB* polypeptide, namely deletion of the first 11 amino acids, caused a number of changes in kinetic responses of this mutant ATCase. However, the deletion of these amino acids at the N-terminal region of *PyrB* polypeptide did not prevent the truncated polypeptide from assembling into an active dodecamer.

Velocity-substrate plots for the wild type ATCase, varying either aspartate or carbamoylphosphate, gave Michaelis-Menten kinetics. In the aspartate saturation curve (Fig 15), it has been shown that all of the effector nucleotides (ATP, CTP, and UTP) inhibited the activity of the enzyme. A significant decrease (98% at 5mM of aspartate) in the specific activity of ATCase was observed when ATP was used as an effector molecule. Therefore, the enzyme is sensitive to ATP inhibition. In carbamoylphosphate saturation curves (Fig 16) with 20mM final concentration of aspartate, it was clear that CTP and UTP activated the enzyme. In other words, an increase in the specific activity was observed when CTP and UTP were used as effector molecules. On the other hand, ATP showed no effect on the enzyme activity. But when the final concentration of

aspartate was set at 1.5mM, the K_m all of the effector molecules inhibited the enzyme (Fig17). Saturated aspartate therefore alters effector binding capacity.

Parallel, kinetic studies were carried out the mutant ATCase. Velocity-substrate plots, varying either aspartate concentrations or carbamoylphosphate concentrations, also gave Michaelis-Menten kinetics. But due to the deletion of the 11 amino acids at the N-terminus of PyrB polypeptide the mutant response to effectors was altered. In aspartate saturation curves (Fig 18), the mutant ATCase was inhibited by ATP and UTP with CTP having no effect at all on the activity of the enzyme until 20 mM of aspartate concentration reached. This would suggest that the binding site for CTP has been modified due to the deletion of the first 11 amino acids at the N-terminus. When a different concentration of carbamoylphosphate was used in carbamoylphosphate saturation (Fig 19) curves, the same results were observed from these curves in which ATP and UTP inhibited the activity of the enzyme, while CTP showed no effect on the enzyme activity. As a results, from both saturation curves (aspartate and carbamoylphosphate) it can be said that the CTP binding site has been altered in the mutant ATCase. This deletion of the 11 amino acids at the N-terminus caused a change in the kinetic responses of this mutant ATCase but it did not prevent the assembly of an active dodecamer.

Purified ATCase from both *P. putida* wild type and mutant type had an apparent molecular mass of 484 kDa as shown by nondenaturing gel electrophoresis that was stained for ATCase activity. Thus, the wild type and the mutant assembled into active dodecamers. Wild-type ATCase and the mutant ATCase were exposed to 42°C and 62°C to see if heat stability were effected in the mutant. However, results showed no

significant difference between wild type and mutant. The mutant appears as one band suggesting no breakdown products (Fig 14).

SDS-polyacrylamide gel electrophoresis was run on the *P. putida* wild type and on the mutant ATCase (Fig 13). The two polypeptides with molecular mass of 36 kDa that assembled from *pyrB* and 44 kDa that assembled from *pyrC* were seen in this gel (Fig 13).

In conclusion, this study concerned the effector response of the ATCase from a wild type *P. putida* and from a mutant ATCase with 11 amino acids deleted at the N-terminus of *PyrB* polypeptide. Results from this study show that wild-type ATCase was more than 90% inhibited by ATP, CTP and UTP at physiological concentrations of carbamoylphosphate and aspartate. The truncated mutant ATCase was shown to be inhibited by ATP 83% and UTP 58% with no effect by CTP at the physiological concentration of each. Thus, the deletion of the first 11 amino acids at the N-terminus significantly altered the binding of effectors. The N-terminus is therefore important in the allosteric regulation of *P. putida* ATCase. These results are in accordance with those of others (9, 32).

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